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14. ABSTRACT

Current therapy still fails to kill migrating (metastasizing) breast cancer cells. Metastatic migration of breast cancer cells is an immediate concern for breast cancer patients as it remains the actual cause of morbidity and mortality. The expression of chemokine receptors, CXCR4, is tightly correlated with the metastatic properties of breast cancer cells. CHK showed its ability to regulate CXCR4 mRNA, supporting our hypothesis that CHK signaling axis may regulate the metastatic migration of breast cancer cells. Especially, wild-type SH2 domain, SH2-R147A and SH2-G129A displayed their differential capacity to regulate CXCR4 expression in breast cancer cells. These results suggest the possibility that the differential binding capacity of CHK SH2 domain to ErbB2 may play a role in CXCR4-mediated breast cancer metastasis. While it still needs further studies to check if CHK gene is mutated in metastatic breast cancer cells, a correlation between CHK and CXCR4 protein was observed in metastatic breast cancer specimens (n=28).

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Introduction

CXCR4 and Metastatic Breast Cancer

Current therapy still fails to kill migrating (metastasizing) breast cancer cells. Metastatic migration of breast cancer cells is an immediate concern for breast cancer patients as it remains the actual cause of morbidity and mortality. The expression of chemokine receptors, such as CXCR4 and CCR7, is tightly correlated with the metastatic properties of breast cancer cells (Muller et al., 2001). This study by Muller et al showed that the level of CXCR4 is higher in malignant breast tumors than in their normal healthy counterparts, suggesting that its expression level correlates with increased metastasis-associated mortality. In vivo, neutralizing the interaction of CXCR4/CXCXL12 significantly impaired the metastasis of breast cancer cells and cell migration (Muller et al., 2001). Furthermore, Kao et al. (Kato et al., 2003) have shown that the expression of CXCR4 in surgically resected invasive ductal carcinomas (n=79) is significantly correlated with the degree of lymph node metastasis. Another study has also described that breast cancer cells metastasized to the lungs express very high levels of CXCR4 as compared with the parental cells, suggesting the pivotal role of CXCR4 in breast cancer cell metastasis (Helbig et al., 2003). These results are further substantiated by the fact that CXCR4 is one of the few genes that is upregulated in bone-metastasized breast cancer cells (Kang et al., 2003). Consistent with these studies, knockdown of endogenous CXCR4 gene expression in breast cancer cells resulted in significant inhibition of breast cancer cell migration in vitro (Chen et al., 2003).

Taken together, CXCR4/CXCL12 signaling axis is a major driving force behind the metastatic migration of breast cancer cells and might be an attractive target for future therapy for metastatic breast cancer. However, CXCR4 is expressed on the surface of several tissues and there have been some concerns about using drugs to target CXCR4. Non-tissue specific blocking of CXCR4 deficient mice displayed serious heart and artery problems suggesting that antagonizing the signaling activity of CXCR4 might have severe side effects in vivo (Schober et al., 2006). In fact, AMD-3100, a CXCR4 receptor blocker, exerted unfavorable side effects in the clinical trial developing gastrointestinal side

effects, thrombocytopenia, and atrial and ventricular arrhythmias and the study was stopped (Hendrix et al., 2004). Accordingly, the CXCR4 blockers are not allowed to be used in the clinical practice and the drug is not likely to move forward in its current formulation. Therefore, it is necessary to develop a strategy to interfere CXCR4/CXCL12 signaling axis selectively in target tissues, such as cancer cells.

Csk homologous kinase(CHK) and CXCR4

Activation and dysregulation of signaling pathways often leads to tumorigenesis. Of signaling pathways, protein tyrosine kinase activity is closely associated with tumor progression and malignancy. Abnormalities of tyrosine phosphorylaton can lead to hyper-proliferative disorders, such as cancer and lymphoma. Csk homologous kinase (CHK) is a non-receptor tyrosine kinase and a second member of the Csk family. Like Csk, CHK has Src homology 2 (SH2) and SH3 domains and lacks the consensus tyrosine phosphorylation and myristylation sites found in Src family kinases. CHK has been shown to phosphorylate the C-terminal negative regulatory tyrosine residue of Src family kinases (e.g. Lck, Fyn, c-Src and Lyn) in vitro and in a yeast co-expression system suggesting that CHK may share functional properties with Csk (Bougeret et al., 2001a). Despite its structural similarities, CHK displays unique molecular functions. While CHK knockout mice didn't show any apparent abnormal phenotype, Csk KO mice showed a defect in the neural tube formation and died at E11.5. (Hamaguchi et al., 1996).

CHK has been suggested to play a specific role as a novel negative growth regulator of human breast cancer on the basis of the following observations: 1) Unlike Csk, which is ubiquitously expressed, CHK is specifically expressed in primary breast cancer specimens, but not in normal breast tissues (Bougeret et al., 2001b; Zrihan-Licht et al., 1998; Zrihan-Licht et al., 1997). 2) Unlike Csk, which cannot associate with ErbB-2, CHK binds directly to phospho-Tyr1248 of the ErbB-2/neu receptor kinase upon heregulin stimulation and inhibits Src kinase activity (Zrihan-Licht et al., 1997). 3) Substantial evidence supports a role for CHK as a negative growth regulator of human breast cancer through inhibition of ErbB-2/neu-mediated Src-family kinase activity. 4) The tumor growth of wild type CHK-transfected breast cancer cells in nude mice is significantly inhibited as compared to that of the non-transfected cells or cells transfected with kinase-dead CHK (Bougeret et al., 2001b). 5) Overexpression of CHK in breast cancer cells markedly inhibits the cell growth, transformation, and invasion induced by heregulin, and also causes a significant delay of cell entry into mitosis (Bougeret

et al., 2001b). Thus, CHK not only inhibits breast cancer cell proliferation and transformation but also may inhibit tumor cell invasion, suggesting its possible role in cell motility and metastasis in breast cancer.

Our previous results showed that CHK regulates CXCR4 expression (Lee et al., 2005). Because CHK is preferentially expressed in breast cancer cells but not in normal breast cells (70 out of 80 breast carcinoma specimens) (Bougeret et al., 2001a), it is anticipated that inhibiting or enhancing CHK activity modulate CXCR4 expression selectively in breast cancer cells and may facilitate the development of side-effect free treatment for metastatic breast cancer.

Body

Task 1. To assess the role of CHK kinase activity in metastatic migration of breast cancer cells using xenograft model:

a. ***Assess CXCR4 expression in wild type (wt) CHK- and dead kinase (dk) CHK-transduced breast cancer cells***

Among the breast cancer cell lines tested to date, MCF-7 and MDA-MB361 breast cancer cells showed a good correlation between the expression of CHK and CXCR4 mRNA expression (Figure 1); A dead kinase full length CHK (called dkCHK hereafter) induced CXCR4 mRNA. Based on these preliminary data, we selected two cell lines, MDA-MB361 and MCF-7, for the further study. We determined whether CHK regulates the cell surface expression of the functional CXCR4 receptors in MCF-7 and MDA-MB361 cells. Whereas dkCHK overexpression induced CXCR4 mRNA, we couldn't observe a significant correlation between CXCR4 mRNA and CXCR4 protein expression in MCF-7 cells (data not shown). While we found a relatively good correlation between CXCR4 mRNA and protein in MDA-MB361 cells, they grow very slowly upon CHK-transduction, which makes us difficult to obtain enough cells for the xenotransplantation experiments. Therefore, to perform *in vivo* experiments as proposed in Task 1b and Task 2c, we established another CHK-expressing breast cancer cell line. A newly established cell line, CHK-transduced SKBR-3, grew at a faster rate than CHK-transduced MDA-MB361 cells. However, CHK-transduced SKBR-3 cells did not show a statistically significant correlation between CXCR4 mRNA and protein levels (data not shown). These

results suggest that the expression of CXCR4 mediated by CHK is subjected to differential post-transcriptional processing and regulation, depending on cell line.

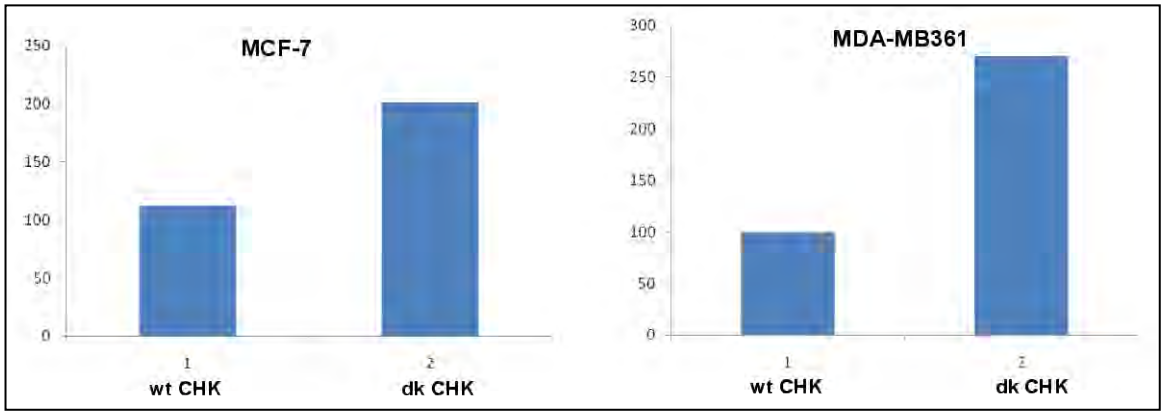


Figure 1. ***Expression profiles of CXCR4 in CHK expressing breast cancer cell lines***

Total RNA was isolated from the indicated cell lines. Aliquots of cDNA were amplified with human CXCR4 and human GAPDH primer sets. The CXCR4 expression was normalized to GAPDH by subtracting their Ct values from the GAPDH Ct value. Values are based on delta-delta-Ct values and represent the median percentages compared to the wild-type CHK value (basal set to 100%). wt CHK: wild-type CHK-transduced cells, dk CHK: dead kinase CHK-transduced cells.

b. ***Transplant wtCHK- and dkCHK-transduced breast cancer cells into female NOD/SCID mice***

NOD/SCID IL-2γ null mice were bred in our own animal facility. Due to the lack of cell lines which show a good correlation between CXCR4 mRNA and protein, we did not perform the xenotransplantation experiments with full length WT CHK and dk CHK-transduced breast cancer cell lines. However, we transplanted SH2 domain CHK-transduced breast cancer cells into NOD/SCID IL-2γ null mice, and the results are now shown in Task 2.c.

c. ***Assess for the presence of metastasis at 7-8 weeks post transplantation and track the transplanted breast cancer cells using imaging system***

As mentioned above, CXCR4 mRNA and protein do not change in parallel in full length CHK transduced breast cancer cell lines. Therefore, metastatic properties of full length CHK transduced breast cancer cell lines were not assessed. However, we performed metastasis assay with SH2 domain CHK transduced breast cancer cell lines as shown in Task 2.C. In addition, we also undertook a correlative study to explore the relationship between the expression of CHK and CXCR4 in biopsy

specimens from metastatic breast cancer patients (n=28). We found a good correlation (R^2 = 0.6208) between CHK and CXCR4 expression in metastatic breast cancer specimens (Figure 2), although it is not clear yet if *CHK* gene in human metastatic breast cancer specimens is mutated.

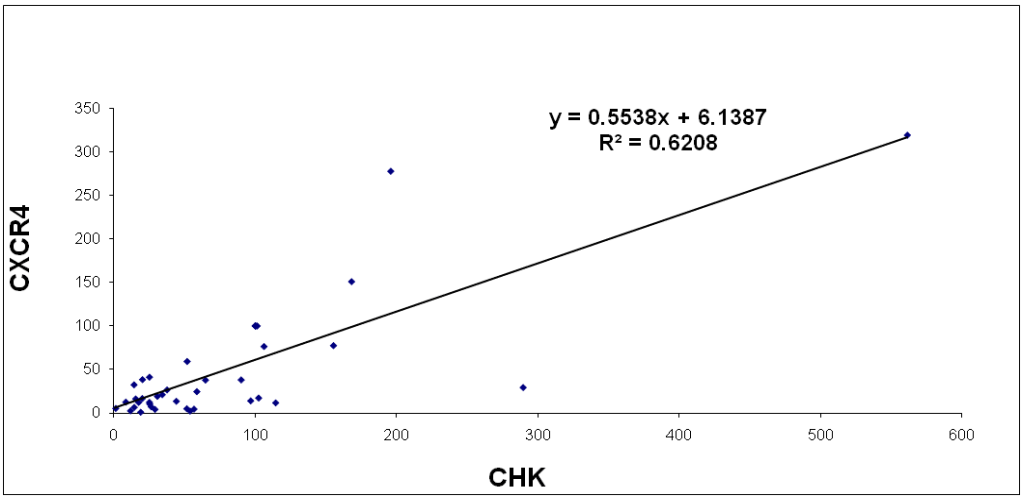


Figure 2. ***A correlation plot between CHK and CXCR4 expression***

Protein lysates from frozen breast cancer tissues (n=28) were analyzed by Western blot for the expressions of CXCR4 and CHK. Protein expression levels were quantitatively estimated by densitometric analysis and normalized to β-actin. To standardize and correct the density among films, the standard sample was loaded on each gel. Data points for each tissue specimen were plotted as a scatter plot.

Task 2. To assess the ability of CHK kinase enhancer/inhibitor to modulate YY1 binding to CXCR4 promoter and CXCR4-mediated breast cancer cell migration

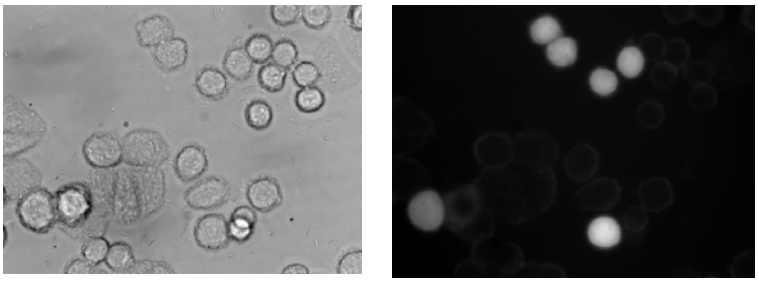
a. Test whether CHK SH2 domain binding mutants, G129R and R147A regulate CXCR4 expression

We successfully completed construction of MSCV-retroviral vectors encoding either wild-type CHK or kinase-dead CHK or wild type SH2 domain or SH2-R147A or SH2-G129A. All these constructs were confirmed by DNA sequencing. When breast cancer cells were transduced with these retroviral constructs, high levels of GFP expression were exhibited in cells, indicating high efficiency of transduction (Figure 3a).

SH2-R147A-transduced cells expressed a significantly higher level of CXCR4 mRNA compared with cells control cells. Furthermore, the level of CXCR4 mRNA in G129R-transduced cells was

lower than that of control and R147A-transduced cells (Figure 3b, left). To verify the cell surface expression of CXCR4 in CHK-transduced cells, we performed flow cytometry analysis on CHK SH2 mutant transduced cells. Retroviral expression of G129R (enhanced binding to ErbB2) decreased the level of CXCR4 protein expression in MCF-7 cells (Figure 3b, right). In contrast, R147A (disrupted binding to ErbB2)-transduced breast cancer cells showed increased level of CXCR4 (Figure 3b, right). These results support our working hypothesis that the differential binding capacity of CHK SH2 domain to ErbB2 may play a role in CXCR4-mediated breast cancer metastasis. It also indicates that truncated forms of the CHK act as more potent regulators of CXCR4 probably because their small size may allow for improved penetration into breast cancer cells.

a.



b.

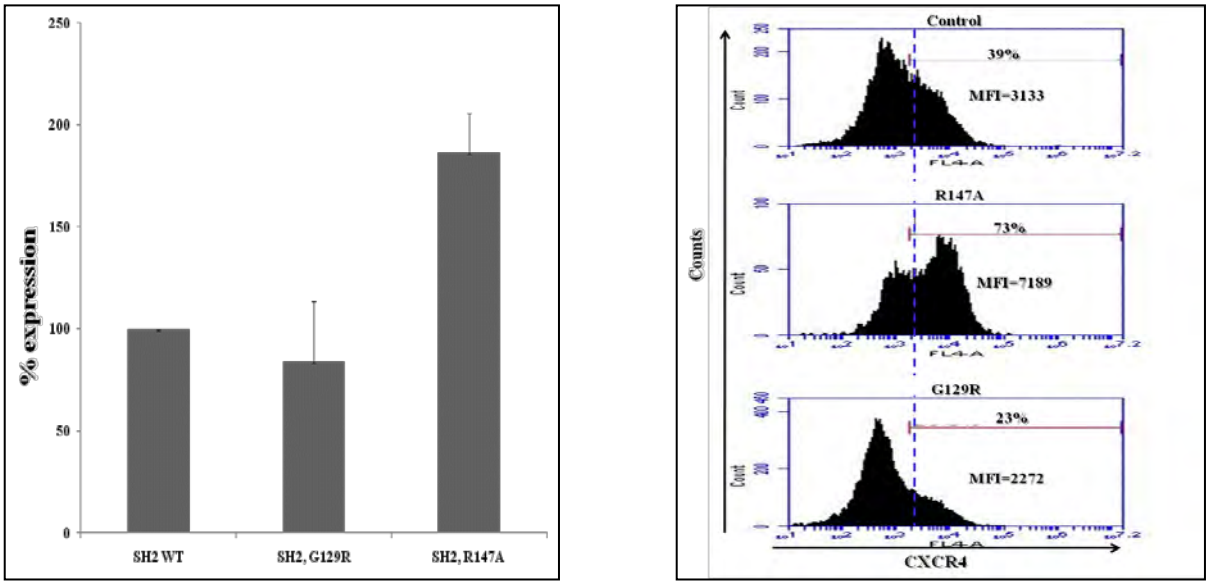


Figure 3. ***Expression of SH2 domain CHK constructs modulates CXC4 expression in breast cancer cells***

a. SKBR-3 breast cancer cells were transduced with MSCV-IRES-GFP-based retroviruses expressing wild-type SH2 CHK. Representative phase contrast (upper left panel) and *GFP*-expressed (upper right panel) images were photographed, b. Left:

Total RNA was isolated from the indicated cells. Aliquots of cDNA were amplified with human CXCR4 and human GAPDH primer sets. The CXCR4 expression was normalized to GAPDH by subtracting their Ct values from the GAPDH Ct value. Values are based on delta-delta-Ct values and represent the median percentages compared to the wild-type CHK value (basal set to 100%); Right: Representative flow cytometry plots showing CXCR4 expression in control (parental MCF-7), R147A, and G129R-transduced MCF-7 cells. Numbers indicate the percentage of CXCR4 positive cells and MFI values.

b. Investigate whether G129R and R147A exert their effects through the transcription factor YY1

We have optimized gel shift assay conditions. We will continue to investigate whether YY1 binding to the CXCR4 promoter is associated with the SH2 mutant CHK-mediated CXCR4 regulation.

c. Perform in vivo metastasis assay using G129R- and R147A-infected breast cancer cells and assess metastasis

Our ultimate aim for this proposal is to investigate whether mutant CHKs modulate CXCR-4 mediated metastasis *in vivo*. We found CHK mutants modulate the expression of CXCR4 (Figure 3b, right). We thus transplanted (i.v. injection) CHK SH2 mutants-transduced MCF-7 cells into NOD/SCID IL-2γ null mice and the lungs of recipient animals were examined 6 weeks post-transplantation. Transplantation of G129R transduced MCF-7 cells resulted in a decreased number of foci in the recipient animals' lung (n=3). Meanwhile, we observed a moderate increase in the number of lung foci in animals (n=3) R147A transduced MCF-7 cells, compared with animals (n=3) transplanted with control MCF-7 cells (mock-transduced). Of note, donor cell marker (GFP) was not detectable by flow cytometry, when the lungs of recipient animals were analyzed. This is probably because of the limited sensitivity of flow cytometry analysis. Nevertheless, a further investigation would be necessary to confirm the identity of cells forming metastatic foci.

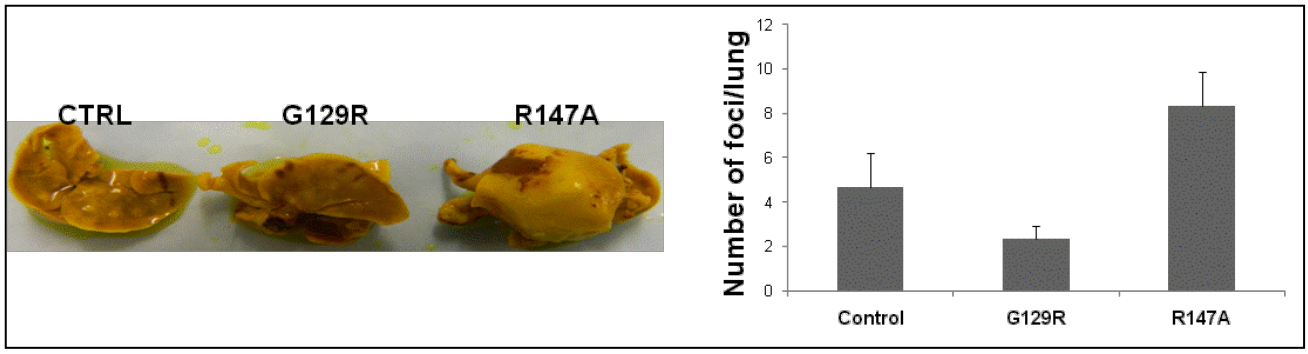


Figure 4. Analysis of metastatic foci in animals that transplanted with SH2 mutants transduced MCF-7 cells.
Lungs were fixed in Bouin's fixative 6 weeks after transplantation. Animals (n=3/group) were sacrificed and the lungs of recipient animals were examined histologically for microscopic metastases. Representative pictures in recipient mice are shown (left). The number of foci was counted and is shown on the y axis (right). Data is expressed as mean ± s.d.

Key Research Accomplishments

1. Our preliminary results indicate that CHK expression regulates the level of CXCR4 in breast cancer cell. Thus signaling pathways downstream of CHK and their cross talk are anticipated to control metastatic migration of breast cancer cells.

2. Our ongoing efforts have generated retroviral vectors carrying wild type (wt) and mutated (mt) CHK (wild-type SH2 domain, SH2-R147A and SH2-G129A) in which eGFP protein is bicistronically expressed as a marker.

3. Lack of a correlation between CXCR4 mRNA and protein in some breast cancer cell lines suggest that CXCR4 are regulated at the post-transcriptional level depending on cell type or cellular context.

4. Differential binding capacity of CHK SH2 domain to ErbB2 may play a role in CXCR4-mediated breast cancer metastasis.

5. Truncated forms of the CHK (SH2 CHKs) act as more potent regulators of CXCR4 probably because their small size may allow for improved penetration into breast cancer cells.

6. A correlation between CHK and CXCR4 protein expression was observed in human primary metastatic breast cancer specimens, although it is not clear yet if *CHK* gene in primary samples is mutated.

Reportable Outcomes

We anticipate publishing a manuscript in near future regarding the role of CHK in CXCR4-mediated breast cancer cell migration, which will depend on the results from the further studies, such as gel-shift assay.

While carrying out the proposed projects, heregulin, which we proposed to regulate CXCR4 expression in breast cancer cells, exerted a potent cytotoxic effect in combination with PPAR gamma agonist in several breast cancer lines. We reported this in Journal of Biological Chemistry.

Park BH, Lee SB, Stolz DB, Lee YJ, Lee BC. 2011. Synergistic interactions between heregulin and PPAR gamma in breast cancer cells. *Journal of Biological Chemistry*, 286: 2087-99.

Conclusion

CHK showed its ability to regulate CXCR4 mRNA, supporting our hypothesis that CHK signaling axis may regulate the metastatic migration of breast cancer cells. Especially, wild-type SH2 domain, SH2-R147A and SH2-G129A displayed their differential capacity to regulate CXCR4 expression in breast cancer cells. Their small size may allow for improved penetration into breast tumor tissue. These results suggest the possibility that the differential binding capacity of CHK SH2 domain to ErbB2 may play a role in CXCR4-mediated breast cancer metastasis. While it still needs further studies to check if *CHK* gene is mutated in metastatic breast cancer cells, a correlation between CHK and CXCR4 protein was observed in metastatic breast cancer specimens (n=28).

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Appendices

N/A